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Growth, photosynthesis, nitrogen partitioning and responses to CO_2 enrichment in a barley mutant lacking NADH-dependent nitrate reductase activity

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Plant growth, photosynthesis and leaf constituents were examined in the wild-type (WT) and mutant *nar1* of barley (*Hordeum vulgare* L. cv. Steptoe) that contains a defective structural gene encoding NADH-dependent nitrate reductase (NADH-NAR). In controlled environment experiments, total biomass, rates of photosynthesis, stomatal conductance, intercellular CO_2 concentrations and foliar non-structural carbohydrate levels were unchanged or differed slightly in the mutant compared with the WT. Both genotypes displayed accelerated plant growth rates when the CO_2 partial pressure was increased from 36 to 98 Pa. Total NADH-NAR activity was 90% lower in the mutant than in the WT, and this was further decreased by CO_2 enrichment in both genotypes. Inorganic nitrate was greater in the mutant than in the WT, whereas in situ nitrate assimilation by excised leaves was two-fold greater for the WT than for the mutant. Foliar ammonia was 50% lower in the mutant than in the WT under ambient CO_2. Ammonia levels in the WT were decreased by about one-half by CO_2 enrichment, whereas ammonia was unaffected by elevated CO_2 in mutant leaves. Total soluble amino acid concentrations in WT and mutant plants grown in the ambient CO_2 treatment were 30.1 and 28.4 μmol g^{-1} FW, respectively, when measured at the onset of the light period. Seven of the twelve individual amino acids reported here increased during the first 12 h of light in the ambient CO_2 treatment, leading to a doubling of total soluble amino acids in the WT. The most striking effect of the mutation was to eliminate increases of glutamine, aspartate and alanine during the latter half of the photoperiod in the ambient CO_2 treatment. Growth in elevated CO_2 decreased levels of total soluble amino acids on a diurnal basis in the WT but not in mutant barley leaves. The above results indicated that a defect in NADH-NAR primarily affected nitrogenous leaf constituents in barley. Also, we did not observe synergistic effects of CO_2 enrichment and decreased foliar NADH-NAR activity on most N-containing compounds.

Abbreviations – C_i, intercellular CO_2; DAS, days after sowing; g_s, stomatal conductance; NADH-NAR, NADH-dependent nitrate reductase; NAD(P)H-NAR, bispecific NADH/NADPH-dependent nitrate reductase; SLW, specific leaf weight; S/R, shoot/root; VPD, vapor pressure deficit; WT, wild-type.

Introduction

Prior to incorporation into organic molecules, nitrate is taken up by roots and converted to ammonia through sequential reactions performed by nitrate reductase (NAR) and nitrite reductase, respectively. NADH-specific and NAD(P)H-bispecific isozymes of NAR have been described in barley (Dailey et al. 1982) and in other species (Wilkinson and Crawford 1993). The barley NAR isozymes are encoded by separate loci having different substrate affinities, and the pH optima varies (Dailey et al. 1982). The NADH-specific enzyme normally is expressed in wild-type (WT) leaf tissue, whereas bispecific NAD(P)H-NAR principally is present in roots of the WT plants (Sueyoshi et al. 1995, Warner and Kleinhofs 1981).

Much has been learned about nitrate assimilation using genetic mutants (Hoff et al. 1994). Several barley mutants that lack NAR activity have been selected from the barley cultivar, Steptoe (Warner et al. 1977). The *nar1* mutation occurs within the structural gene encoding the catalytic subunit of NADH-dependent NAR (NADH-NAR). Owing to the presence of bispecific NAD(P)H-NAR, which is synthesized by the *nar7* locus, *nar1* will grow to maturity in the field or when using nitrate as the sole source of inorganic N (Oh et al. 1980). Studies of barley mutants and those of other species indicated that foliar nitrate levels were increased by a deficiency of NADH-NAR activity (Scheible et al. 1997a, Warner and Kleinhofs 1981). It is also known that tobacco mutants with greatly reduced levels of NADH-NAR activity have decreased foliar amino acids (Foyer et al. 1994, Scheible et al. 1997a). However, amino acid levels have not been investigated in barley *nar1* mutants where NADP(H)-NAR activity is present and potentially may alter the findings. Also, with the exception of glutamine (Scheible et al. 1997a), changes of amino acids in NADH-NAR mutants have not been examined on a diurnal basis.

Rising atmospheric CO_2 concentrations can impact plants both in natural and in managed ecosystems. The majority of plants exposed to elevated CO_2 exhibited enhanced growth rates, and this created an increased demand for nutrients from the soil. For reasons that are not entirely clear, rates of N uptake and assimilation may be inadequate when plants are grown under elevated CO_2 (Conroy and Hocking 1993). Consequently, CO_2-enriched plants often have an increased C/N ratio, decreased inorganic and total N per unit dry mass and increased N use efficiencies in comparison to plants grown in ambient CO_2 (Stitt and Krapp 1999). Plants raised under elevated CO_2 often display symptoms of N deficiency. This can include a buildup of leaf starch, decreased soluble protein, decreased Rubisco activity and Chl(a + b) breakdown similar to that observed during senescence (Sicher and Bunce 1997, Stitt and Krapp 1999). Growth in elevated CO_2 decreased foliar nitrate but did not alter amino acid levels in well-fertilized WT plants (Geiger et al. 1999). However, the combined effects of CO_2 enrichment and of a deletion of NADH-NAR activity on amino acid metabolism have not been investigated in detail.

Numerous lines of evidence have shown that NADH-NAR is a highly regulated enzyme and that it has a role in coordinating C and N metabolism in plants (Campbell 1999). We hypothesized that a deficiency of NADH-NAR activity would decrease rates of amino acid and protein synthesis. This change in leaf composition should alter rates of photosynthesis and of plant growth. Plants grown with high rates of N fertility generally do not show large acclimation responses to CO_2 enrichment (Geiger et al. 1999). Therefore, we postulated that the *nar1* mutant would have reduced N assimilation rates and a modified response to CO_2 enrichment in comparison to the WT.

Materials and methods

Plant material

Barley (*Hordeum vulgare* L. cv. Steptoe) and *nar1* (GSHO 2413), a mutant derived from the parent line lacking NADH-NAR activity, was obtained from the National Small Grains Collection (USDA, Aberdeen, ID) through the Germplasm Resources Information Network (http://www.ars-grin.gov). This mutant was induced by azide mutagenesis and was isolated and characterized by Warner et al. (1977). Cultivar Steptoe contains dormancy factors that can completely inhibit or delay seed germination. Therefore, prior to planting, all seeds were imbibed in the dark on filter paper for 3–5 days at 6°C. Seeds were then kept overnight at room temperature in the dark, and any seed that failed to sprout was manually dehusked. Two to three of the pretreated seeds were planted in 1.5-l pots containing a 1:1 mixture of vermiculite and a sphagnum-based soil amendment (Pro-Mix Professional; Premier Horticulture, Inc., Quakertown, PA). Plants were raised in matching controlled environment chambers (model M-2; Environmental Growth Chambers Corp., Chagrin Falls, OH) equipped with high-intensity discharge lamps and 10 Sunbrella reflectors (Environmental Growth Chambers Corp., Chagrin Falls, OH) provided by the manufacturer (Sicher 1997). Plants were grown on a 14-h day/10-h night cycle using an air temperature of 22 ± 1°C and an irradiance of 700 ± 30 μmol m^{-2} s^{-1}. Chamber air CO_2 partial pressures were either 36 ± 2.1 (ambient) or 98 ± 3.3 Pa (elevated) and were maintained as described previously (Sicher 1997). Approximately 7 days after sowing (DAS), plants were thinned to one plant per pot. Plants usually

were harvested 21 or 35 DAS either within 1 h of midday or at indicated times. For dry matter determination, plants were separated into shoot and root fractions, and these were dried in a forced air oven at 70°C for 3 days prior to weighing. Plants were watered on alternate days for the first 10 DAS and then daily thereafter using a complete mineral nutrient solution (Robinson 1984).

Photosynthesis measurements

Net CO_2 assimilation rates (A) were measured 3–4 h after the start of the light period using a portable IR gas analysis system (Ciras-1; PP Systems, Haverhill, MA). Measurements were performed 34 and 35 DAS on two recently, fully expanded collared leaves from individual plants. Gas exchange rates were determined at 36 and 98 Pa CO_2 for plants grown both at ambient and at elevated CO_2. These were the A(36) and A(98) measurement conditions, respectively. Gas exchange measurements were performed in the same controlled environment chambers used for plant growth. The irradiance was 1500 μmol m^{-2} s^{-1}, PPF (photosynthetic photon flux) density, temperature was 22°C and vapor pressure deficit (VPD) was less than 1.0 kPa. Other measurement details are given elsewhere (Sicher and Bunce 2001).

Leaf components

Leaf constituents were measured using plants raised either in the glasshouse or in the growth chambers. A single 5-cm long leaf segment was removed from the midsection of the most recently, fully expanded collared leaf. For quantifying in vivo nitrate assimilation, entire leaves were excised from the plant, and the leaf ends were cut a second time under H_2O. Excised leaves of all three genotypes were incubated in the dark at 22°C with the cut ends immersed in deionized H_2O for 0, 6 and 24 h. Sampled leaves or leaf segments were quickly sealed in paper envelopes and placed in liquid N_2 to quench metabolism. Frozen leaf tissue was extracted immediately or was stored at −80°C until use.

Immediately prior to extraction, leaf material was ground to a liquid N_2 powder using a mortar and pestle, and 0.1–0.2 g FW was transferred to a ground glass tissue homogenizer on ice. Soluble carbohydrates were extracted with organic solvents and analyzed using coupled enzyme assays (Sicher and Bunce 2001). Total Chl(a + b) was measured in 80% acetone as described by Lichtenthaler (1987). Soluble protein was extracted in dilute buffer and quantified colorimetrically with Coomassie Brilliant Blue R-250 (Bio-Rad Labs, Hercules, CA). Inorganic nitrate was extracted from leaf tissue using 80% ethanol. Extracts were diluted 1:50 with deionized H_2O and microfiltered as described below, and nitrate was determined by HPLC using an anion exchange column (Thayer and Huffaker 1980). Foliar ammonia was determined according to Kun and Kearney (1974). Approximately 50 mg of leaf tissue was homogenized in 1 ml of 5% TCA at 0°C as described above. The extract was spun in a microcentrifuge at 10 000 g for 5 min, and the supernatant was neutralized with 2 M $KHCO_3$. Neutralized extracts were stored in tightly sealed cryovials at −20°C for up to 1 month. Reactions were performed at 25°C in a total volume of 1 ml that contained 0.25 ml neutralized extract, 100 mM Tris–HCl, pH 8.0, 10 mM 2-oxoglutarate, 0.2 mM NADPH and 5 units of glutamic dehydrogenase from *Proteus* sp. (G4387; Sigma-Aldrich, St Louis, MO). Decreased absorbance was determined after 0 and 240 s using a UV-2101-PC spectrophotometer (Shimadzu Corp., Columbia, MD), and reactions were initiated with enzyme. Controls contained 0.25 ml H_2O in lieu of extract.

Soluble amino acids were determined by an HPLC procedure using the AccQTag method (Waters Corp., Milford, MA). Leaf tissue (0.1 g FW) was homogenized at room temperature with 2 ml of 70% methanol. The homogenate was incubated for 15 min at 45°C in a H_2O bath and then centrifuged for 15 min at 5800 g. The pellet was washed with 1 ml of 70% methanol, the supernatants were combined and the extracts were evaporated to dryness under a stream of N_2 at 37°C. The dried samples were resuspended in 0.5 ml of 20 mM HCl and then centrifuged through a 0.22 μm Ultrafree-MC membrane filter unit (Millipore Corp., Bedford, MA). Tissue extracts and standards were derivatized with the AccQFluor kit from Waters according to the manufacturer's instructions. Separations were performed using a Waters 600E Multisolvent Delivery System equipped with a 3.9 × 150 mm AccQTag C_{18} column from the same manufacturer. The elution gradient was based on recommendations in the AccQFluor kit except that the running buffer was adjusted to a pH of 5.80. Detection was with a Shimadzu RF-535 fluorimeter using excitation and emission wavelengths of 250 and 395 nm, respectively. The output of the detector was monitored using Empower2 software from Waters. Quantification was based on four-point standard curves prepared with standard amino acids from Pierce Chemicals (Rockford, IL), and α-aminobutyric acid served as an internal standard. Because of incomplete chromatographic separations, alanine and valine contained small amounts of arginine and cysteine, respectively.

Enzyme activities

The activity of NADH-NAR from barley leaf extracts was measured at 25°C using an in vitro colorimetric procedure

as described previously (Sicher 2001). Measurements were performed in the absence of Mg^{2+} to determine total NADH-NAR activity (Huber et al. 1992), and reaction mixtures contained 50 m*M* HEPES–NaOH (pH 7.5), 5 m*M* KNO_3, 3 m*M* EDTA, 5 μ*M* flavin adenine dinucleotide, 0.2 m*M* NADH and 0.2 ml of leaf extract in a total volume of 0.5 ml. Assays were terminated after 5 min in a boiling H_2O bath, and the NO_2^- formed during the reaction was detected as a diazo compound formed with sulfanilamide (Aslam et al. 1979). Blanks were prepared with leaf extracts kept in boiling H_2O for 2 min prior to assay.

Statistics

Significant differences were determined with a two-way analysis of variance procedure using StatView 5.0 for Windows (SAS Institute, Cary, NC).

Results

Plant growth responses

Total biomass accumulation for *nar1* and the WT was 1.9 and 2.6 g ($P \leq 0.01$), respectively, when plants were grown for 35 days in the 36 Pa CO_2 treatment (Table 1). Both the mutant and the WT exhibited a positive growth response ($P \leq 0.01$) when the atmospheric CO_2 partial pressure was increased from 36 to 98 Pa. The net increase in biomass attributed to CO_2 enrichment for *nar1* and the WT was 1.9 and 1.5 g, respectively. Therefore, total biomass of the WT and of the mutant was 4.1 ± 0.2 and 3.9 ± 0.2 g, respectively, when measured 35 DAS in the elevated CO_2 treatment. In a related greenhouse experiment using ambient CO_2, total biomass of the mutant was 17% lower than that of the WT 56 DAS, and the anthesis date was delayed 7–14 days for the mutant compared with the WT (data not shown). Shoot/root (S/R) ratios measured 35 DAS did not differ between genotypes ($P > 0.05$) in this investigation. However, S/R ratios for both genotypes increased by 45% ($P \leq 0.01$) on average in response to CO_2 enrichment. Specific leaf weight (SLW) was significantly lower for *nar1* than the WT ($P \leq 0.01$), and SLW of both genotypes was increased by CO_2 enrichment ($P \leq 0.01$).

Leaf constituents and enzyme activities were measured using leaf segments of 21- or 35-day-old plants raised in controlled environment chambers at either ambient or elevated CO_2. Starch, sucrose, glucose and fructose did not differ by genotype ($P > 0.05$) when grown in the ambient CO_2 treatment, and levels of these non-structural carbohydrates increased ($P \leq 0.01$) in response to CO_2 enrichment. Both soluble protein and Chl(a + b) were lower ($P \leq 0.01$) in leaves of *nar1* vs the WT, but neither leaf constituent was affected by CO_2 enrichment when results for both genotypes were combined ($P > 0.05$). However, a genotype by CO_2 interaction was detected for Chl(a + b), suggesting that elevated CO_2 affected

Table 1. Growth parameters, gas exchange rates and leaf carbohydrate levels for WT barley and the *nar1* mutant. Individual leaves were harvested from plants grown in controlled environment chambers for 21 days at ambient (36 Pa) and elevated (98 Pa) CO_2. Values are means ± SE for n = 6–10. *Significant differences at $P \leq 0.05$. **Significant differences at $P \leq 0.01$. Leaf starch values are in μmol glucose equivalents.

	WT		*nar1*		*P*		
Parameter	36 Pa	98 Pa	36 Pa	98 Pa	CO_2	Genotype	Interaction
Biomass (g)	2.6 ± 0.2	4.1 ± 0.2	1.9 ± 0.1	3.9 ± 0.2	**	*	NS
S/R ratio	2.2 ± 0.1	2.9 ± 0.2	1.9 ± 0.1	3.0 ± 0.2	**	NS	NS
SLW (g DW m^{-2})	60.3 ± 2.4	76.8 ± 2.2	51.7 ± 3.1	68.7 ± 2.8	**	**	NS
Chl(a + b) (mg g^{-1} FW)	1.1 ± 0.05	0.94 ± 0.03	0.87 ± 0.04	0.92 ± 0.03	NS	*	*
Protein (mg g^{-1} FW)	46.8 ± 2.5	42.2 ± 0.6	36.3 ± 1.4	37.4 ± 2.2	NS	*	NS
Ammonia (μmol g^{-1} FW)	1.2 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	NS	*	**
A(36) (μmol m^{-2} s^{-1})	26.4 ± 0.8	22.4 ± 1.4	24.4 ± 1.8	16.4 ± 0.9	**	**	NS
A(98) (μmol m^{-2} s^{-1})	37.6 ± 1.2	37.0 ± 0.9	35.2 ± 0.9	30.8 ± 1.2	*	**	NS
g_s 36 (mmol m^{-2} s^{-1})	394 ± 41	264 ± 22	365 ± 17	201 ± 15	**	NS	NS
g_s 98 (mmol m^{-2} s^{-1})	363 ± 31	220 ± 13	350 ± 15	189 ± 15	**	NS	NS
Ci 36 (μl l^{-1})	228 ± 6	198 ± 5	239 ± 6	213 ± 7	**	*	NS
Ci 98 (μl l^{-1})	742 ± 15	649 ± 12	752 ± 6	661 ± 16	**	NS	NS
Sucrose (μmol g^{-1} FW)	35.1 ± 1.2	73.2 ± 3.7	40.8 ± 1.9	63.1 ± 3.3	*	NS	*
Glucose (μmol g^{-1} FW)	2.9 ± 0.5	3.8 ± 0.6	2.6 ± 0.4	3.8 ± 1.0	NS	NS	NS
Fructose (μmol g^{-1} FW)	4.0 ± 0.5	5.7 ± 0.5	3.6 ± 0.4	4.7 ± 0.9	*	NS	NS
Starch (μmol g^{-1} FW)	25.3 ± 1.8	59.3 ± 1.2	27.9 ± 1.3	59.3 ± 2.8	**	NS	NS
NADH-NAR (nmol g^{-1} FW s^{-1})	7.0 ± 0.3	4.9 ± 0.9	0.96 ± 0.1	0.12 ± 0.1	*	**	NS

Chl(a + b) differently in the WT than in the mutant. A genotype by CO_2 interaction also was detected for ammonia ($P \leq 0.01$). The simplest explanation for this was that ammonia levels for WT barley were about twice as high in the ambient than in the elevated CO_2 treatment. In comparison, ammonia was unaffected by CO_2 enrichment in leaves sampled from *nar1*. Total NADH-NAR activity in leaf tissue of the WT was about 7 nmol g^{-1} FW s^{-1}, and this was reduced 29% by CO_2 enrichment. Total NADH-NAR activity in the mutant was 90% lower than that of the WT ($P \leq 0.05$), and this enzyme activity was further decreased by the elevated CO_2 treatment. Consequently, NADH-NAR activity differed significantly ($P < 0.05$) among the genotype and CO_2 treatments.

Leaf gas exchange

Leaf gas exchange rates were determined using 34- to 35-day-old plants raised in controlled environment chambers with continuous treatments of 36 and 98 Pa CO_2. Genotypic differences ($P \leq 0.01$) in net rates of A were observed for both A(36) and A(98) measurements (Table 1). Growth at 98 Pa CO_2 decreased A(36) for both genotypes. Although the magnitude of this reduction was less for the WT in comparison to *nar1*, the interaction between genotypes was not significant at $P > 0.05$. The same statistical result occurred for the A(98) measurement, but the reduction in net CO_2 uptake because of growth under elevated CO_2 was less for A(98) than for A(36). Regardless of the growth CO_2 treatment, A(36) measurements were 6% lower for *nar1* compared with the WT. Similar percentage reductions for *nar1* were observed in the A(98) measurements. Genotypic differences were detected for stomatal conductance (g_s) ($P \leq 0.05$). In this study, g_s was strongly influenced by the growth CO_2 treatment, and values of g_s for plants grown in the ambient and elevated CO_2 treatments did not respond to the A(36) and A(98) measurement conditions. Under both measurement CO_2 conditions, intercellular CO_2 (C_i) was lower ($P \leq 0.01$) for the elevated compared with the ambient CO_2-grown mutant line in comparison to the WT.

In vivo nitrate assimilation

Consistent with a loss of NADH-NAR activity, foliar nitrate levels were greater ($P \leq 0.01$) in mutant *nar1* in comparison to the WT (Fig. 1). Immediately after leaf excision, nitrate levels were 150 ± 16 and 225 ± 11 μmol g^{-1} FW in WT and *nar1* leaves, respectively. Nitrate levels in excised WT leaves decreased 45% when incubated in the dark for 6 h with the cut ends in deionized H_2O. In comparison, leaf nitrate amounts in excised leaves of *nar1* remained unchanged ($P > 0.05$) over the same time period. However, 61 and 29% of the nitrate that was originally present in leaves of the WT and *nar1*, respectively, was assimilated 24 h after excision.

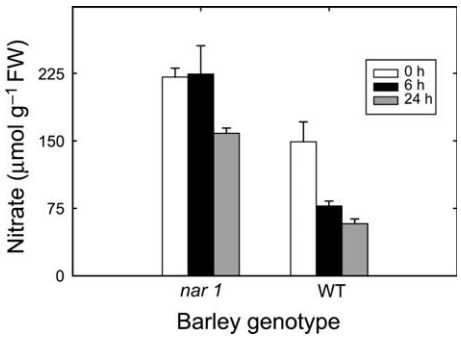

Fig. 1. In situ nitrate assimilation by excised leaves of WT and barley mutant, *nar1*, with defective NADH-NAR activity. Nitrate levels were determined at 0 (white), 6 (black) and 24 h (gray) after leaf excision. Excised leaves were incubated with cut ends immersed in distilled H_2O in the dark at 22°C. Values are means ± SE.

Soluble amino acids

Changes of soluble amino acids in mutant and WT leaves were quantified using 21-day-old plants grown under ambient and elevated CO_2 in controlled environment chambers. In this experiment, results for up to12 individual amino acids are shown using alcohol extracts of barley leaf tissue (Figs 2 and 3). In addition, leaves were harvested 0, 6 and 12 h after the beginning of the light period. The most abundant soluble amino acids detected in barley leaves were aspartic acid, glutamic acid, glutamine, serine and glycine (Fig. 2A, C–F, respectively). One amino acid, glutamic acid, comprised greater than 30% of the total soluble amino acid pool under all treatment conditions. Total soluble amino acids were calculated by summing the concentrations of the 12 individual amino acids presented here along with that of seven others that are not shown. When examining leaves of WT plants grown under ambient CO_2, aspartic acid, glutamine, serine, glycine, alanine (Fig. 3A), threonine (Fig. 3C) and valine (Fig. 3B) were low at the beginning of the photoperiod and increased in almost a linear fashion for the next 12 h. In contrast to the above, glutamic acid, asparagine, proline and the minor constituent amino acids leucine and phenylalanine exhibited little or no accumulation in the WT throughout the photoperiod in the ambient CO_2 treatment. Total soluble amino acid concentrations in WT plants grown in the ambient and elevated CO_2 treatments were 30.1 and 23.6 μmol g^{-1} FW, respectively, when measured at the first sampling.

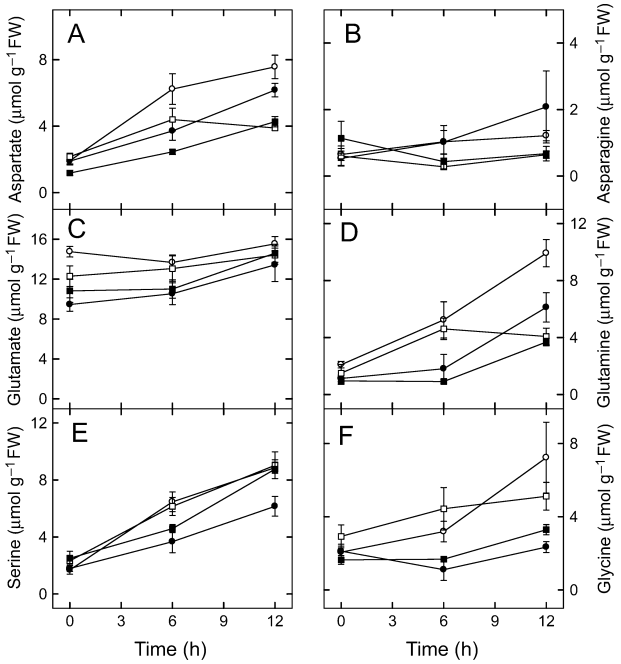

Fig. 2. Effects of NADH-NAR deficiency and CO_2 enrichment on diurnal changes of soluble amino acids in leaves of the WT and barley mutant *nar1*. WT (○, ●) and mutant *nar1* (□, ■) plants were grown in controlled environment chambers for 21 days under ambient (36 Pa; ○, □) and elevated (98 Pa; ●, ■) CO_2. Soluble amino acids were quantified using the AccuTag procedure. Values are means ± SE for n = 6–10.

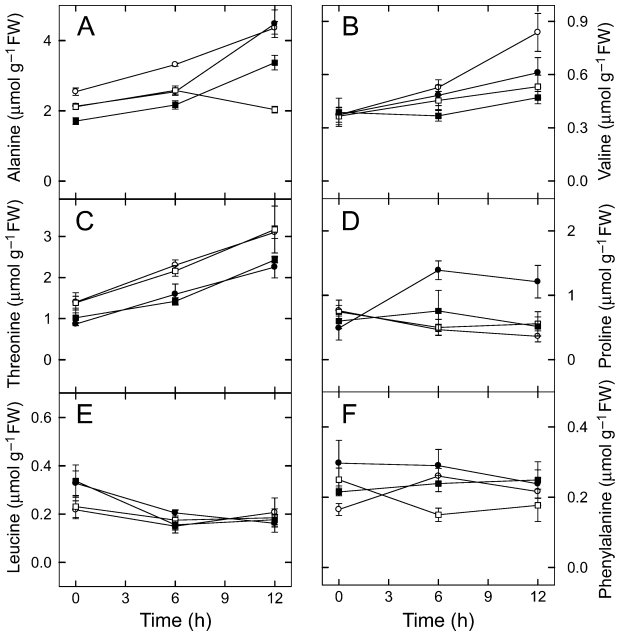

Fig. 3. Effects of NADH-NAR deficiency and CO_2 enrichment on diurnal changes of soluble amino acids in leaves of WT and barely mutant *nar1*. Conditions, symbols and other experimental details were as in Fig. 2.

Comparable values for the mutant were 28.4 and 24.3 μmol g^{-1} FW, respectively, when measured under the above conditions. When all measurement time points were combined, CO_2 enrichment decreased levels of aspartic acid, glutamic acid, glutamine, serine, valine and threonine in WT leaves. Interestingly, CO_2 enrichment increased levels of proline but did not alter asparagine or leucine concentrations in this genotype.

The presence of a mutation within NADH-NAR altered concentrations of some but not all individual amino acids in barley leaves, and this was strongly dependent on diurnal factors. Summing the levels of individual soluble amino acids in the WT and *nar1* revealed that total concentrations were 60.9 and 46.0 μmol g^{-1} FW, respectively, when measured at the 12-h light time point using plants grown in the ambient CO_2 treatment. Total soluble amino acid level in mutant *nar1* was 45.4 μmol g^{-1} FW at the 12-h sampling in the elevated CO_2 treatment. Consequently, CO_2 enrichment did not affect total soluble amino acid levels in mutant leaves. Concentrations of specific amino acids in *nar1* leaves were decreased at the end of the photoperiod in the ambient CO_2 treatment. This was readily observed for aspartic acid, glutamine and alanine. However, at the same time, and also in the ambient CO_2 treatment, glutamic acid, serine, glycine, threonine and leucine were similar in leaves of the WT and *nar1*.

Discussion

Nitrate uptake and assimilation by higher plants is a complex and highly regulated process that is controlled by various transcriptional and posttranslational mechanisms (Tischner 2000). Nitrate serves as the primary source of N for many higher plants and is a powerful signaling metabolite that is involved in the induction of several proteins including those that function in nitrate transport and assimilation (Crawford 1995). High nitrate concentrations also decrease photosynthetic starch accumulation in favor of sucrose synthesis (Scheible et al. 1997b). It is believed that this shift to sucrose biosynthesis increases organic acid levels, which then serve as substrates for assembling amino acids.

Growth and CO_2 assimilation

Based on results of the current study, mutant *nar1* attained nearly normal growth 35 DAS. This finding was largely in agreement with prior results by Warner and Kleinhofs (1981) using the same barley genotypes as used here. These results also supported prior studies of NADH-NAR mutants and related transformants from other species including *Arabidopsis thaliana* (Wilkinson and Crawford

1993), *Nicotiana plumbaginifolia* (Gabard et al. 1987) and *Nicotiana tabacum* (Scheible et al. 1997a, Vaucheret et al. 1989).

Total NADH-NAR activity was reduced by about 90% in the mutant compared with the WT. The residual NADH-NAR activity in leaves of mutant *nar1* previously has been attributed to the presence of a bispecific NADP(H)-NAR that functions with either NADH or NADPH as substrate (Dailey et al. 1982). Also, this residual NADH-NAR activity potentially explains the nearly normal growth of the mutant in comparison to the WT (Oh et al. 1980). Furthermore, the total biomass of both genotypes increased in response to CO_2 enrichment, indicating that the low levels of bispecific NAD(P)H-NAR activity in the *nar1* mutant were present in sufficient amounts to support the enhanced growth rates that occurred in elevated CO_2 environments. The observation that inducible NADH-NAR activity in higher plant leaves is present in excessive amounts is not new (Wilkinson and Crawford 1993). Because this enzyme is very highly regulated and performs an important metabolic function, the ability of mutants to grow in its absence has been a major conundrum in plant biology (Crawford 1995).

In the current study, total NADH-NAR activity was decreased by CO_2 enrichment ($P < 0.05$) both in the mutant and in the WT. An earlier report also showed that total NADH-NAR activity decreased in response to elevated CO_2 using barley primary leaves from a different cultivar (Sicher 2001). In this earlier study, NADH-NAR activity was similar early in the photoperiod but decreased in the elevated compared with the ambient CO_2 treatment several hours later. The residual NAR activity in the mutant, which accounted for about 10% of the total present in the WT under ambient CO_2, was further decreased by CO_2 enrichment. This may indicate that NAD(P)H-NAR activity in barley was decreased by CO_2 enrichment. Alternatively, the residual NAR activity in *nar1* may be because of the fact that the NADH-NAR isozyme was not completely abolished by the mutation.

Rates of A(36) and A(98) by *nar1* were reduced 6–8% in comparison to the WT when plants were grown in the ambient CO_2 treatment. Therefore, net rates of CO_2 assimilation were only slightly altered by a genetic lesion in the NADH-NAR structural gene alone. Foliar nitrate assimilation is an energy intensive process and consumes a total of eight electrons during the conversion of nitrate to ammonium. If this process were interrupted, a feedback inhibition of A would occur. Therefore, it is likely that bispecific NAD(P)H-NAR and possibly other reductive processes served as alternate sinks for electrons in the *nar1* mutant. Note that bispecific NAD(P)H-NAR activity is normally confined to roots in the WT but its activity is induced or, at a minimum, revealed in leaves of the mutant lines (Sueyoshi et al. 1995). The fact that rates of A differed by less than 10% may explain why differences in biomass between WT and *nar1* in the current study were small when plants were in the early growth stages but became more evident with increased plant age (see above; Warner and Kleinhofs 1981).

Large decreases of A(36) compared with A(98) because of growth at 98 Pa CO_2 is a common pattern of acclimation to elevated CO_2. According to Sage (1994), this result was because of greater changes in Vc (rate of Rubisco carboxylation) in comparison to J_{max} (rate of photosynthetic electron transport). Reductions in g_s for both WT and *nar1* because of growth at 98 Pa CO_2 indicated that an acclimation of g_s occurred in response to growth CO_2. Because C_i was only affected slightly, this was likely the result of a parallel acclimation of g_s and A (Sage 1994). The finding that g_s did not respond to the measuring CO_2 partial pressure may be because of low VPD (Bunce 1998). Also, the lack of a stomatal response to measurement CO_2 may be unique to cultivar Steptoe because it was not observed under similar growth conditions employing a different barley genotype (Sicher 1998) or with soybean grown under field conditions (Leakey et al. 2006).

Leaf components

Overall, some leaf components were affected by a deletion of NADH-NAR activity, and others were little changed. Major carbohydrate storage pools in leaves did not differ by genotype under either CO_2 treatment. This was consistent with the observation that A was only slightly lower in the mutant compared with the WT at least during early vegetative growth. As expected, all the carbohydrates measured here except glucose were increased by CO_2 enrichment. Although a CO_2 by genotype interaction was observed for sucrose, this did not alter the overall conclusions.

In contrast to soluble non-structural carbohydrates, inorganic nitrate concentrations in foliar tissue were greater in the mutant than in the WT. This finding was reported previously for NADH-NAR mutants in barley and for other species (Scheible et al. 1997a, Warner and Kleinhofs 1981) and is consistent with decreased NADH-NAR activity in the mutant. The amount of nitrate assimilated by excised WT leaves over a 24-h time period was about twice that observed for *nar1*. This was consistent with the decreased NADH-NAR enzyme activity in the mutant compared with the WT.

Foliar ammonia was decreased by CO_2 enrichment in the barley WT and by decreased NADH-NAR activity in the *nar1* mutant in comparison to the WT. However, CO_2

enrichment did not decrease ammonia levels in leaves of the *nar1* mutant. Decreases of ammonia in response to CO_2 enrichment have been observed previously and were likely the result of decreased rates of photorespiration (Scheible et al. 1997a, Sicher 2001). Reduced ammonia concentrations in the mutant were also expected because of diminished NADH-NAR activity because ammonia is the end product of nitrate reduction. Because ammonia levels were not further decreased by CO_2 enrichment in the *nar1* mutant, it may indicate that a minimum level of ammonia in the leaf is necessary for normal metabolism. Because ammonia is incorporated into organic acids to synthesize soluble amino acids, low levels of ammonia in the mutant, and in the WT under CO_2 enrichment, could have important implications for amino acid metabolism and subsequent protein synthesis.

Foyer et al. (1994) and Scheible et al. (1997a) previously reported that tobacco plants engineered to express limited amounts of NADH-NAR contained decreased levels of amino acids, particularly glutamine. Because *N. plumbaginifolia* contains a single gene encoding NAR activity, these results were expected. A summation of prior investigations into the responses of soluble amino acids to CO_2 enrichment is more complicated. Geiger et al. (1999) reported that amino acid levels in mature leaves of 35-day-old tobacco plants were unaffected by CO_2 enrichment when plants were N sufficient but that large decreases occurred when the N supply was restricted. For soybean plants grown in the field, Rogers et al. (2006) concluded that amino acid levels were decreased by CO_2 enrichment early in the growing season but that treatment differences disappeared later in the year when the plants were more robust. Members of the same research group (Ainsworth et al. 2007) subsequently reported that amino acids were increased by CO_2 enrichment in developing soybean leaves, but differences were not observed in fully expanded leaves. This was in agreement with results for tobacco showing that CO_2 enrichment increased amino acids in young leaves of tobacco seedlings (Geiger et al. 1999).

Combined effects of CO_2 enrichment and decreased NADH-NAR activity on individual amino acids were determined in the current study at three time points during the photoperiod. Results showed that responses of individual amino acids to a mutation within NADH-NAR and to CO_2 enrichment varied markedly. These differing responses undoubtedly indicated that specific individual amino acids in barley leaves were subject to stringent regulation within divergent or possibly separate biochemical pathways. With the exception of glutamic acid, concentrations of amino acids in barley leaves were generally low at the beginning of the photoperiod. Seven of the 12 individual amino acids reported here increased substantially during the first 12 h of light in the ambient CO_2 treatment. As a result, total soluble amino acids in WT barley leaves essentially doubled in concentration over the first 12 h of the photoperiod in the ambient CO_2 treatment. Both CO_2 enrichment and a deletion of NADH-NAR activity decreased total soluble amino acid levels in barley leaves. However, CO_2 enrichment did not further decrease total soluble amino acid levels in leaves of *nar1*.

Both glycine and serine were decreased by CO_2 enrichment, but overall differences were generally greater for glycine than serine. Ainsworth et al. (2007) obtained similar results with soybean leaves and suggested that effects of elevated CO_2 on glycine and serine were because of an inhibition of the photorespiratory pathway. These authors also argued that serine was less affected by CO_2 enrichment than glycine because of its potential involvement in non-photorespiratory metabolism.

One of the most interesting observations in this paper was that levels of glutamine, aspartic acid and to a lesser extent alanine decreased during the latter half of the photoperiod in mutant leaves in the ambient CO_2 treatment. Therefore, levels of these three amino acids in the *nar1* mutant under ambient CO_2 were about half that of the WT when determined after 12 h of light. This late-day decline was not observed for the other amino acids reported here. It is not known why changes of glutamine and aspartic acid occurred in the mutant late in the day. However, a similar large decrease of glutamine levels late in the photoperiod occurred in a separate study using barley primary leaves. However, reduced diurnal glutamine concentrations in the latter study were in response to CO_2 enrichment rather than decreased NADH-NAR activity (unpublished results).

In summary, we examined the effects of CO_2 enrichment on WT barley and on a mutant line deficient in NADH-NAR activity. The mutant only differed slightly from the WT in terms of growth A and non-structural carbohydrate content. In spite of reduced NADH-NAR activity in leaves, growth of the mutant was accelerated in response to CO_2 enrichment. Mutant leaves differed from the WT in terms of overall N metabolism. The *nar1* mutant contained increased nitrate levels and decreased rates of in vivo nitrate consumption by excised leaves. Moreover, total soluble amino acids, ammonia, Chl(a + b) and soluble protein were all decreased in the mutant genotype compared with the WT. A marked decline in glutamine, aspartic acid and alanine occurred under ambient CO_2 conditions during the last half of the photoperiod in mutant barley leaves compared with the WT. The elevated CO_2 treatment also reduced amino acid levels in WT barley leaves. However, synergistic effects of

decreased NADH-NAR activity and CO_2 enrichment on soluble amino acid concentrations in barley leaves were not observed.

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